MOLECULAR AND CELLULAR BIOLOGY, June 1995, p. 2942–2954 Vol. 15, No. 6
0270-7306/95/$04.00+0

Site-Specific Initiation of DNA Replication in *Xenopus* Egg Extract Requires Nuclear Structure

DAVID M. GILBERT,† HIROSHI MIYAZAWA,‡ AND MELVIN L. DEPAMPHILIS*

Roche Institute of Molecular Biology, Roche Research Center, Nutley, New Jersey 07110-1199

Received 16 December 1994/Returned for modification 30 January 1995/Accepted 3 March 1995

Previous studies have shown that *Xenopus* egg extract can initiate DNA replication in purified DNA molecules once the DNA is organized into a pseudonucleus. DNA replication under these conditions is independent of DNA sequence and begins at many sites distributed randomly throughout the molecules. In contrast, DNA replication in the chromosomes of cultured animal cells initiates at specific, heritable sites. Here we show that *Xenopus* egg extract can initiate DNA replication at specific sites in mammalian chromosomes, but only when the DNA is presented in the form of an intact nucleus. Initiation of DNA synthesis in nuclei isolated from G_1-phase Chinese hamster ovary cells was distinguished from continuation of DNA synthesis at preformed replication forks in S-phase nuclei by a delay that preceded DNA synthesis, a dependence on soluble *Xenopus* egg factors, sensitivity to a protein kinase inhibitor, and complete labeling of nascent DNA chains. Initiation sites for DNA replication were mapped downstream of the amplified dihydrofolate reductase gene region by hybridizing newly replicated DNA to unique probes and by hybridizing Okazaki fragments to the two individual strands of unique probes. When G_1-phase nuclei were prepared by methods that preserved the integrity of the nuclear membrane, *Xenopus* egg extract initiated replication specifically at or near the origin of bidirectional replication utilized by hamster cells (dihydrofolate reductase ori-β). However, when nuclei were prepared by methods that altered nuclear morphology and damaged the nuclear membrane, preference for initiation at ori-β was significantly reduced or eliminated. Furthermore, site-specific initiation was not observed with bare DNA substrates, and *Xenopus* eggs or egg extracts replicated prokaryotic DNA or hamster DNA that did not contain a replication origin as efficiently as hamster DNA containing ori-β. We conclude that initiation sites for DNA replication in mammalian cells are established prior to S phase by some component of nuclear structure and that these sites can be activated by soluble factors in *Xenopus* eggs.

Origins of DNA replication in animal viruses and single-cell eukaryotic organisms consist of specific *cis*-acting sequences that function independently (autonomously replicating sequences) when transferred to the appropriate cells or cell extract (25, 26, 58). However, attempts to identify similar sequences in multicellular eukaryotes have resulted in a paradox: while DNA synthesis is initiated at specific, genetically determined sites in cellular chromosomes, identification of *cis*-acting DNA sequences that control replication has proven elusive.

Mapping initiation sites for DNA replication at 17 different locations in the chromosomes of mammals and flies has revealed that DNA synthesis initiates not randomly throughout cellular chromosomes but at specific DNA sites. These sites consist of a primary initiation locus referred to as the origin of bidirectional replication (OBR) surrounded by many secondary initiation loci distributed throughout a larger initiation zone. From 80 to 95% of DNA synthesis occurs bidirectionally from the OBR. This conclusion is based on the fraction of replication forks traveling in the same direction as determined by two-dimensional (2D) neutral/alkaline gels (64, 83), the ratio of Okazaki fragments that hybridize to the two strands of a unique DNA probe (6, 15, 20, 54, 88), and the ratio of long leading nascent DNA strands from forward arms of replication forks that hybridize to the two strands of a unique DNA probe (16, 46, 54, 57). In addition, quantitative analysis of specific DNA sequences within long nascent DNA strands reveals that most of them originate bidirectionally from a small chromosomal locus (39) and that this locus can reside within an initiation zone (97).

Most OBRs are contained within as little as 0.5 kb to as much as 3 kb (references 4, 6, 26, 39, 64, 87, 88, 91, and 95 and references therein), although some OBRs lie within larger regions of 5 to 11 kb (36, 46, 54, 83). The fact that these OBRs have been identified by independent investigators using a variety of different methods gives confidence that site-specific initiation is not an artifact of the experimental conditions used to map them. Similar results were obtained with synchronized and unsynchronized cells, with cells containing single-copy sequences and with cells containing amplified multicopy sequences, with cells treated and not treated with metabolic inhibitors, and by using different methods for detecting specific nascent DNA sequences.

Analyses of DNA structures by 2D neutral/neutral gel electrophoresis at five different genomic locations detected replication bubbles throughout a larger initiation zone of 6 to 55 kb that includes the OBR (30, 31, 64, 66, 83). These results are consistent with those cited above if one assumes that the frequency of initiation events at the OBR is much greater than the frequency of initiation events outside the OBR. In fact, replication bubbles detected by 2D neutral/neutral gel analyses appear more abundant in the 12-kb region containing the dihydrofolate reductase (DHFR) ori-β OBR in CHO cells (30, 31) and in the 8-kb region at the 5′ end of the human rRNA transcription unit (66), where subsequent nascent DNA strand

* Corresponding author. Mailing address: Roche Institute of Molecular Biology, Roche Research Center, 340 Kingsland St., Nutley, NJ 07110-1199. Phone: (201) 235-2428. Fax: (201) 235-2839. Electronic mail address: depamphm@rnisd0.dnet.roche.com.

† Present address: Department of Biochemistry and Molecular Biology, SUNY Health Sciences Center at Syracuse, Syracuse, NY 13210.

‡ Present address: The Institute of Physical and Chemical Research (RIKEN), Wako, Saitama 351-01, Japan.

analysis revealed a greater than 10-fold excess of newly synthesized DNA relative to other sites within the initiation zone (97). The relative number of initiation events in different DNA segments is difficult to quantify by 2D gel analysis (30, 31, 59, 66). In contrast, analyses of labeled nascent DNA chains reveal the preference for one site relative to another. For example, the ratio of DNA synthesis between the two templates of a specific DNA fragment automatically provides the minimum fraction of replication forks moving in the same direction through this region. Initiation events distributed randomly outside the OBR simply contribute to the background level in these mapping methods.

Metazoan replication origins are determined at least in part by specific DNA sequences. First, the same origins that are utilized in cells containing single copies of a unique genetic locus are also utilized in cells containing hundreds of copies of the same locus (20, 26, 54, 91). Second, some origins have been found to retain their activity when translocated to other chromosomal sites (46, 80), and origin activity near the human β-globin gene is eliminated by an 8-kb deletion (57). Third, several recent reports of autonomously replicating sequence elements that function in mammalian cells and cell extracts have been documented in detail and shown to correspond to initiation sites for DNA replication in mammalian chromosomes (6, 87, 91, 95). Nevertheless, DNA replication can initiate within virtually any DNA sequence under some conditions for transfecting cultured mammalian cells with plasmid DNA (22, 59, 68) or when bare DNA is introduced into *Xenopus* eggs or egg extract (22). Moreover, replication within the chromosomes of rapidly dividing cells in *Xenopus* and *Drosophila* embryos appears to begin at randomly chosen sites (50, 82).

One solution to these paradoxical results is that origins of DNA replication in metazoan chromosomes are determined by nuclear structure in addition to DNA sequence. Newly synthesized DNA is preferentially bound to some component of nuclear structure generally referred to as nuclear matrix or nuclear scaffold (52) and is organized into discrete replication factories (49). Replication of DNA introduced into either *Xenopus* eggs or egg extract does not occur unless DNA is first assembled into nuclei containing nuclear lamins and functional nuclear pores (9, 23, 53, 78). Once formed, the nuclear envelope is instrumental in regulating the onset of S phase, apparently by regulating access of chromosomal DNA to one or more initiation factors (licensing factor) present in the cytoplasm (22).

To determine whether site-specific initiation of DNA replication requires nuclear structure, bare DNA or nuclei isolated from hamster cells were incubated in a *Xenopus* egg extract, and initiation of DNA replication was examined in the vicinity of a well-defined OBR (ori-β) that resides downstream of the DHFR gene in CHO cells. The same origin is also active in CHOC 400 cells, a derivative of CHO cells that contains about 1,000 tandemly integrated copies of a 243-kb sequence surrounding the DHFR gene (63), substantially increasing the ability to detect initiation events. Results presented here demonstrate that site-specific initiation of DNA replication can be achieved in a cell-free system under conditions previously thought to initiate replication exclusively by a nearly random selection of initiation sites. Soluble factors in *Xenopus* egg extract can initiate DNA replication in intact nuclei from G_1-phase cells at or close to DNA sites chosen by the hamster cell. Moreover, this site specificity required that the DNA be organized in an intact nuclear structure previously assembled in vivo. Together with the results summarized above, these results suggest that mammalian origins of DNA replication consist of a unique organization between specific chromosomal sequences and components of nuclear structure.

MATERIALS AND METHODS

Synchronization of hamster cells. CHOC 400 cells were passaged for four to five generations in medium (Dulbecco's modified Eagle's medium supplemented with nonessential amino acids and 5% fetal calf serum) containing 1 mg of methotrexate (Calbiochem) per ml prior to performing the experiments described in this report in order to ensure that the amplified DHFR locus was still present at high copy number in all of the cells of the population. Homogeneous populations of G_1-phase CHOC 400 cells were obtained by mitotic selection, using a modification of the procedure described by Gilbert and Cohen (40). Flasks (~80% confluent) were tapped vigorously 10 times to remove loosely attached cells, and then the medium was replaced with fresh medium containing 0.05 μg of nocodazole (Calbiochem) per ml to block cells in metaphase. Four hours later, medium from each flask was removed, and the flask was tapped moderately four times to detach mitotic cells that were harvested in their original nocodazole-containing medium at room temperature until all flasks had been completed. The percentage of mitotic cells (90 to 98%) was scored (40), and ~2 × 10^6 cells were stained with propidium iodide and analyzed for DNA content in a fluorescence-activated cell sorter (FACS) (44). Typically, 24 225-cm^2 flasks yielded 8 × 10^7 to 12 × 10^7 mitotic cells.

The remainder of the mitotic cells were transferred to fresh 37°C medium. The progression of cells through G_1 into S phase was monitored by (i) FACS analysis of DNA content, using 2 × 10^6-cell aliquots, (ii) incorporation of 5′-bromodeoxyuridine (BrdU) followed by staining cells with anti-BrdU antibodies, and (iii) incorporation of [^{3}H]Thd into acid-precipitable material. To measure incorporation of precursors, aliquots containing 10^5 cells were seeded into 3-cm tissue culture dishes, and either 2 μCi of [^{3}H]Thd (1 mCi/ml; Amersham) per ml or 33 μg of BrdU (Sigma) per ml was added to the culture medium 30 min before harvesting cells. To determine the fraction of cells in S phase, cells were fixed in 70% ethanol, concentrated to 1 × 10^6 to 2 × 10^6 cells per ml, and dropped onto glass microscope slides. Slides were baked at 65°C for 10 to 15 min, transferred to ice-cold methanol for 10 min and then ice-cold acetone for 30 s, and then incubated in 1.5 M HCl for 30 min and washed twice in phosphate-buffered saline (PBS). Slides were then washed once in PBS containing 0.5% Tween 20 (PBS-Tween 20) for 5 min, and cells were stained with 3 μl of undiluted anti-BrdU antibody (Becton Dickinson) in a humidified chamber for 1 to 2 h at room temperature (~21°C). Slides were washed twice for 5 min in PBS-Tween 20, and cells were stained as before with 3 μl of undiluted fluorescein isothiocyanate-conjugated goat anti-mouse antibody (whole molecule; Sigma) containing 50 to 100 μg of bisbenzimide (Boehringer) per ml. Slides were washed again twice in PBS-Tween 20 and mounted in PBS. Incorporation of [^{3}H]Thd into hamster cell DNA was determined as acid-precipitable radioactivity (40). By these criteria, it was determined that the first cells enter S phase 6 h after mitosis. By 3 to 4 h after release from mitosis, 95 to 99% of the cells had a DNA content of 2N and only 0.2 to 1.0% had initiated DNA replication. Therefore, nuclei isolated from these cells were called G_1 nuclei. By 12 h after mitosis, more than 80% of the cells were actively synthesizing DNA. Therefore, nuclei isolated from these cells were called S nuclei.

Preparation of nuclei. Digitonin nuclei were prepared by lysing cells with digitonin, using a modification of the protocol described by Adam et al. (2). CHOC 400 cells were removed from the dish by trypsinization, counted, washed twice (10^7 cells per ml) in ice-cold transport buffer (2), resuspended in transport buffer (10^7 cells per ml), and then mixed with an equal volume of transport buffer containing 20 μg of digitonin (Calbiochem) per ml. Cells were then incubated on ice for 5 min and washed twice in cold transport buffer. Dounce nuclei were prepared by lysing cells in a Dounce homogenizer as described by Heintz and Stillman (48). Triton nuclei were prepared by mixing Dounce nuclei at 2 × 10^7/ml with an equal volume of hypotonic buffer (48) containing 0.2% Triton X-100 and then incubating them on ice for 5 min. Nuclei were then washed three times in fresh hypotonic buffer. Scraped nuclei were prepared by mechanically scraping cells from a culture dish (70). Sperm nuclei were prepared by demembranating *Xenopus* sperm with lysolecithin and freezing the sperm as described previously (8).

DNA synthesis in nuclei. S-phase Chinese hamster ovary (CHO) cell extract (48), *Xenopus* egg extract (7), and a high-speed supernatant of *Xenopus* egg extract (9) were prepared and handled as described elsewhere. Hamster nuclei, prepared as described above, were resuspended in the designated extract at 25,000 nuclei per μl. Sperm nuclei were added to extracts at 1,000/μl. The fraction of DNA that replicated was determined by supplementing reactions with 25 μCi of [α-^{32}P]dATP (6,000 Ci/mmol; Amersham) per ml and then transferring 3-μl aliquots to 300 μl of stop buffer (0.5% sodium dodecyl sulfate [SDS], 20 mM Tris [pH 8.0], 10 mM EDTA) at the times indicated, measuring acid-precipitable ^{32}P, and calculating the fraction of DNA that replicated as described by Blow and Laskey (8). The fraction of nuclei engaged in DNA synthesis was measured by incorporation of BrdU triphosphate (BrdUTP) and staining as described above. For this purpose, reaction mixtures were supplemented with 250 μM BrdUTP, and at the indicated times, a 1-μl sample was removed, added to 2 μl of hypotonic buffer, and then stained.

DNA synthesis in plasmid DNA. Plasmids were propagated in a *dam*$^+$ strain of *Escherichia coli* in order to fully methylate *Dpn*I restriction endonuclease sites, rendering these molecules sensitive to cleavage by *Dpn*I. Since eukaryotic cells lack this methylase, *Dpn*I restriction sites become hemimethylated after one round of replication in *Xenopus* egg extract (27). Therefore, plasmid DNA (10 ng of DNA per μl of extract) was incubated in *Xenopus* extract at 21°C as described previously (8), and at the indicated times, 3-μl aliquots were transferred to 300 μl of stop buffer. Circular plasmid DNA was isolated (43) and converted into linear monomers by digestion with *Pvu*I, and then half of the sample was digested with *Dpn*I (43). DNA samples were fractionated by electrophoresis in 0.65% agarose gels, transferred to Hybond N+ (Amersham), and hybridized with ^{32}P-labeled pUC19 (common to all three plasmids used), using the hybridization and washing conditions described previously (41). The amount of ^{32}P-pUC19 that hybridized to full-length plasmid DNA was quantified by PhosphorImager analysis, and the fraction that replicated was determined as (*Dpn*I-resistant DNA/2)/(total DNA − *Dpn*I-resistant DNA/2).

The same experiments were also carried out with linear concatemers prepared by linearizing each plasmid (pneoS13 and pDG1a with *Aat*II and pDGλ with *Nco*I) and self-concatemerizing the linear plasmids separately with T4 DNA ligase before cointroducing the plasmids into *Xenopus* egg extract. After replication in extract, isolated linear DNA was remonomerized with the respective restriction enzyme and analyzed as for circular DNA, except that blots were hybridized with probes specific for each plasmid.

Density gradient centrifugation. Reaction mixtures were supplemented with 250 μM BrdUTP and 25 μCi of [α-^{32}P]dATP per ml. At the times indicated, 300 μl of stop buffer was added, and DNA from 5×10^5 nuclei was precipitated with isopropanol and washed twice in 70% ethanol to completely remove unincorporated nucleotides as described below. For neutral pH gradients, DNA samples were resuspended in 135 μl of TE (10 mM Tris [pH 8.0], 1 mM EDTA) and added to 5.2-ml Quick Seal (Beckman) centrifuge tubes. The tubes were filled with a solution of Cs_2SO_4 in TE (refractive index = 1.3715) and centrifuged at 27,000 rpm for 48 h in a Beckman VTi65 rotor. For alkaline pH gradients, DNA samples were resuspended in 200 μl of distilled water, added to tubes that were then filled with a solution of Cs_2SO_4 (1.479 g/ml) in 0.1 M NaOH–10 mM EDTA, and centrifuged at 45,000 rpm for 24 h in a Beckman VTi65 rotor. Fractions were collected from the bottom of the tube, and the amount of radiation per fraction was determined with a scintillation counter. Marker DNA was made in the following manner. CHOC 400 cells were propagated for two generations in the presence of 0.02 μCi of [^{14}C]Thd (50 mCi/mmol; Amersham) per ml to provide light (unsubstituted) DNA. These cells were incubated with 33 μg of BrdU (Sigma) per ml and 3 μCi of [^{3}H]cytidine (20 Ci/mmol; Amersham) per ml for an additional 14 h to provide hybrid DNA in which Thd in one strand is replaced by BrdU.

Plasmids and DNA probes. Each of the probes described in Fig. 6, as well as a negative hybridization control, bacteriophage λ nucleotides (nt) 24508 to 25157, was cloned into the polylinker of pBluescript II KS(+) and pBluescript II SK(−) (Stratagene). Probes H, I, J, G, F, K, and L in this study are equivalent to probes A, B, C, D, E, F, and G, respectively, in reference 46 and were subcloned from plasmids obtained from H. Cedar. Probe B is identical to, and probes A, C, D, and E are similar to, probes of the same name described by Burhans et al. (15). Probe A represents nt 61 to 704 of the DHFR cDNA (71). Probes C, D, E, and R were amplified from cosmid cSc26 (3) by PCR using oligonucleotide primers that created a *Sac*I or *Hin*dIII site at the 5′ ends of nt 2430 to 2650 (C), 2638 to 2919 (D), 3939 to 4319 (E), and 4812 to 5699 (R) of the sequenced DHFR ori-β region (18). Probes P and O were subcloned from plasmids kindly provided by P. Dijkwel and J. Hamlin. Cosmid cSc26 (3) and plasmid pneoS13 (3, 43) have been described previously. pDG1 was constructed by cloning the 7.5-kb *Hin*dIII fragment containing the DHFR promoter and first three exons (19) into pUC19. pDGλ8.6 was constructed by cloning the 6.6- and 2.0-kb *Hin*dIII fragments from bacteriophage λ into pUC19. Plasmid DNA was prepared using Qiagen columns as instructed by the manufacturer.

Mapping origins by the early labeled fragment hybridization method. (i) In cultured cells. Mitotically synchronized CHOC 400 cells were collected at their G_1/S-phase boundary by incubating them in complete medium containing aphidicolin for 14 h and then releasing them into S phase for 3 to 5 min before radiolabeling (15, 27). Nuclei (typically 10^7 per assay) were prepared by lysing these cells in a Dounce homogenizer on ice (48) and collecting the nuclei in 1.5-ml microcentrifuge tubes (5 s, 16,000 × *g*), and the nuclear pellet (~24 μl per tube) was resuspended with 6 μl of ice-cold 5× replication cocktail (150 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES; pH 7.6], 35 mM $MgCl_2$, 4 mM dithiothreitol, 500 μM each dTTP, dGTP, and dCTP, 1 mM each CTP, GTP, and UTP, 20 mM ATP, 200 mM creatine phosphate, 100 μg of creatine phosphokinase per ml) containing 100 μCi of lyophilized [α-^{32}P]dATP (6,000 Ci/mmol). Reaction mixtures were incubated for 2 min at 26°C, the reaction was stopped by adding 470 μl of lysis buffer (50 mM Tris-HCl [pH 7.8], 10 mM EDTA, 0.4 M NaCl, 0.6% SDS, 200 μg of RNase A per ml, 200 μg of proteinase K per ml), and then the mixtures were incubated for 1 h at 37°C before addition of 167 μl of saturated NaCl and 700 μl of isopropanol. DNA was pelleted, washed twice with 70% ice-cold ethanol, and resuspended in 400 μl of TE buffer containing 50 μg of RNase A per ml. The resuspended DNA was reduced to fragments of 200 to 400 bp by sonication. [^{32}P]DNA was precipitated in 0.3 M sodium acetate with 1 volume of isopropanol and resuspended in 2 ml of Church and Gilbert hybridization buffer (21). Typically, about 1 cpm of Cerenkov radiation per nucleus was recovered.

Hybridization was to 1 μg of each double-stranded plasmid DNA immobilized on Hybond N+ (Amersham) membranes, using the procedure described in the EVENFLO capillary slot blot user manual (Laboratory Product Sales, Rochester, N.Y.). Filters were prehybridized in 5 ml of hybridization buffer at 65°C for at least 15 min. Labeled replication intermediates were denatured by heating to 100°C for 5 min and hybridized in 2 ml at 65°C for 20 h. Membranes were washed twice for 15 min at 65°C in 0.2 × SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.2% SDS before exposure to a Molecular Dynamics PhosphorImager cassette for 1 to 3 days. Relative amounts of hybridization to each probe were quantified by using the Image Quant program. Nonspecific hybridization of replication intermediates to the negative hybridization control (see description of DNA probes above) was routinely 10 to 20% of the weakest probe hybridization signal and was subtracted from each template to calculate the relative counts per minute per base pair.

(ii) In *Xenopus* egg extract. G_1 nuclei (typically 10^7 per assay) were incubated in *Xenopus* egg extract for 1 to 3 h in the presence of 100 μg of aphidicolin per ml or for 40 to 45 min in the absence of aphidicolin. Nuclei were then washed two times with 1 ml of ice-cold hypotonic buffer, and replication forks were allowed to briefly regenerate by adding 6 μl of 5× release buffer (same as 5× replication cocktail except that 2.5 μM dATP replaces [α-^{32}P]dATP) and incubating the nuclei for 1 min at 21°C. Nuclei were washed one more time with cold hypotonic buffer, and nascent DNA was labeled as described above except that labeling was for 2 min at 12°C. All subsequent steps were as described above. Typically, about 1 cpm of Cerenkov radiation per nucleus was recovered.

Mapping origins by the Okazaki fragment distribution method. Replication intermediates were labeled as for the early labeled fragment method except that the radiolabeling periods were reduced to 1.5 min for G_1/S-phase cells and 20 s for nuclei incubated in *Xenopus* egg extract. ^{32}P-labeled Okazaki fragments were isolated as described previously (15). Single-stranded DNA probes were prepared by using helper phage VCM13 (Stratagene). Each probe (3 μg) was subjected to electrophoresis in a 1% agarose gel and transferred to Hybond N+ (Amersham). Filters were hybridized with ^{32}P-labeled Okazaki fragments and quantified as described for the early labeled fragment method. Typically, 5×10^7 nuclei produced 1×10^6 to 2×10^6 Cerenkov cpm of purified Okazaki fragments.

G_1 nuclei that initiated replication in *Xenopus* egg extract containing aphidicolin contained nascent DNA chains the size of Okazaki fragments that hybridized equally to both templates of each probe. These fragments likely arose from repair of broken DNA replication forks that accumulate in the presence of aphidicolin and other inhibitors of fork progression (32, 43, 85). This problem was avoided with G_1/S-phase hamster cells by allowing them to briefly enter S phase prior to labeling nascent DNA (15, 27). In *Xenopus* extract, initiation of DNA replication was sufficiently synchronous after only 45 min that use of aphidicolin could be avoided entirely.

RESULTS

DNA synthesis in G_1 nuclei is due to replication and not repair. CHOC 400 cells were synchronized in G_1 phase by mitotic selection (40) at a point 2 to 3 h before the first cells entered S phase. FACS analysis and BrdU labeling of nuclei (see Materials and Methods) revealed that at least 95% of the population had a DNA content of 2N and greater than 99% had not yet initiated DNA replication. When G_1 nuclei were isolated from these cells by the digitonin method (digitonin nuclei) and then incubated in *Xenopus* egg extract, DNA synthesis began after 10 to 30 min of incubation, and by 40 to 60 min, virtually 100% of the nuclei were engaged in replication (see Fig. 3). This early phase of DNA synthesis in G_1 nuclei exhibited four characteristics of chromosomal DNA replication that distinguished it from DNA repair.

The first characteristic was semiconservative DNA replication. G_1 nuclei were incubated in *Xenopus* egg extract containing [α-^{32}P]dATP and BrdUTP. BrdUTP is incorporated in place of Thd, thereby increasing the buoyant density of nascent DNA (27). After 40 min of incubation, genomic DNA was purified and centrifuged to equilibrium in a neutral Cs_2SO_4 density gradient, where virtually all of the [^{32}P]DNA appeared as a single band of hybrid density (Fig. 1A). Since hybrid density DNA could result from either complete synthesis of one DNA strand or small patches of repair in both DNA strands, it was necessary to determine whether heavy DNA was covalently linked to light DNA. Therefore, hybrid-density DNA was isolated from the neutral Cs_2SO_4 gradient and cen-

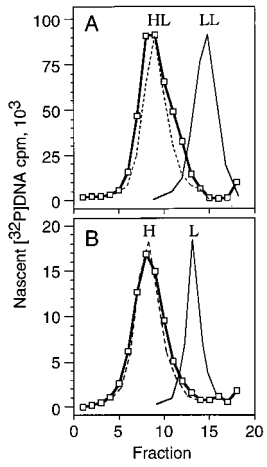

FIG. 1. DNA synthesis induced in G_1 nuclei resulted from semiconservative DNA replication. Nuclei prepared by lysing G_1-phase CHOC 400 cells with digitonin were incubated 40 min in *Xenopus* egg extract supplemented with [α-^{32}P]dATP and BrdUTP. (A) Total genomic DNA was isolated and centrifuged to equilibrium in a neutral Cs_2SO_4 gradient. Gradients were fractionated from the bottom of the tube, and the amount of [^{32}P]DNA per fraction was determined by scintillation counting. (B) Fractions indicated by the shaded region in panel A were pooled, centrifuged to equilibrium in an alkaline Cs_2SO_4 gradient, and analyzed as in panel A. The positions of BrdU-containing chromosomal DNA (heavy [HH or H]) and unsubstituted DNA (light [LL or L]) that were analyzed in parallel gradients with each experiment are indicated. Similar results were obtained with Dounce nuclei and scraped nuclei.

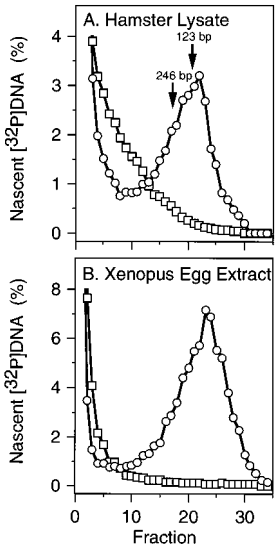

FIG. 2. DNA synthesis induced in G_1 nuclei involved Okazaki fragments. (A) hamster lysate. Nascent DNA was radiolabeled in nuclei isolated from exponentially proliferating CHOC 400 cells with [α-^{32}P]dATP for 1.5 min at 26°C (○). An excess of unlabeled dATP was added to an aliquot of these nuclei, and the incubation continued for 10 min (□). (B) *Xenopus* egg extract. Nuclei isolated from G_1-phase CHOC 400 cells were incubated in *Xenopus* egg extract for 45 min and then pulse-labeled for 20 s at 12°C with [α-^{32}P]dATP (○). An excess of unlabeled dATP was added to an aliquot of these nuclei, and the incubation continued for 10 min (□). The DNA was purified and fractionated by electrophoresis in a 1.8% alkaline agarose gel. The gel was sliced into 5-mm fractions that were used to measure Cerenkov radiation in a scintillation counter, and the percentage of the total counts per minute in each gel slice was calculated. Arrows indicate the positions of molecular weight markers. Similar results were obtained with Dounce nuclei and scraped nuclei.

trifuged to equilibrium in an alkaline Cs_2SO_4 density gradient in order to separate heavy and light strands. Single-stranded ^{32}P-DNA appeared as a unique band of heavy density (Fig. 1B), confirming that DNA synthesis resulted from semiconservative DNA replication.

The second characteristic was that DNA synthesis occurred discontinuously through the repeated synthesis and joining of short nascent DNA chains (Okazaki fragments) on the retrograde arm of replication forks. Okazaki fragments in exponentially proliferating CHOC 400 cells could be labeled by incubating isolated nuclei briefly with [α-^{32}P]dATP and then fractionating the DNA by electrophoresis in alkaline agarose gels. This procedure revealed a population of [^{32}P]DNA chains 50 to 250 nt in length that were rapidly joined to long DNA molecules when the incubation was continued for 10 min in the presence of an excess of unlabeled dATP (Fig. 2A). Similar results were obtained with G_1 nuclei that had been incubated in *Xenopus* egg extract for 40 min before being exposed briefly to [α-^{32}P]dATP (Fig. 2B), although it was necessary to reduce both the time and temperature of the radiolabeling period to accommodate the short half-life of Okazaki fragments synthesized by *Xenopus* enzymes (8). The short, transient nascent DNA chains radiolabeled in either protocol hybridized preferentially to one of the two templates (discussed below). Therefore, DNA synthesis occurred at replication forks.

The third characteristic was sensitivity to inhibitors of DNA polymerases α and δ but not to inhibitors of DNA polymerase β. DNA polymerases α and δ are required for DNA replication, whereas DNA polymerase β is required for DNA repair (92). G_1 nuclei were incubated in *Xenopus* egg extract with increasing amounts of either aphidicolin, cytosine arabinoside triphosphate (araCTP), or ddTTP (data not shown). DNA synthesis was inhibited 50% by either 3 μg of aphidicolin per ml or 5 μM araCTP (>98% by 8 μg of aphidicolin per ml and >95% by 250 μM araCTP) but was completely resistant to 1 mM ddTTP (the ddTTP/dTTP ratio was 20:1), consistent with a dependence on DNA polymerases α and δ but not DNA polymerase β (92).

The fourth characteristic was that DNA synthesis occurred at discrete foci distributed throughout the nucleus (1, 35, 49, 55, 74, 79). When G_1 nuclei were incubated in *Xenopus* egg extract containing BrdUTP and then stained with fluorescein-conjugated anti-BrdU antibodies, discrete replication foci were observed in each nucleus after a delay of about 30 min that were indistinguishable from those observed in early S-phase CHOC 400 cells (data not shown).

***Xenopus* egg extract initiates DNA synthesis de novo in G_1 nuclei.** To verify that DNA synthesis in G_1 nuclei resulted from de novo initiation of nascent DNA chains originating in *Xenopus* egg extract and not from continuation of DNA synthesis at preexisting replication forks, DNA synthesis induced in G_1 nuclei was compared with DNA synthesis in nuclei isolated from S-phase CHOC 400 cells (S nuclei), which contain abundant active replication forks. As a control, DNA synthesis was also examined in demembranated *Xenopus* sperm nuclei, a natural postmeiotic DNA substrate for *Xenopus* egg replication factors in which replication forks are entirely absent. The results demonstrated that *Xenopus* egg extract provides soluble replication factors that are required to initiate DNA synthesis de novo in G_1 nuclei.

S nuclei continued to synthesize DNA when incubated in a cytosolic extract from S-phase CHOC 400 cells, but DNA synthesis did not occur in either G_1 nuclei or sperm nuclei (Fig. 3D). Therefore, DNA synthesis in G_1 nuclei and sperm nuclei required one or more replication factors that were not required by S nuclei. When the same experiment was carried out in *Xenopus* egg extract, DNA synthesis occurred in all

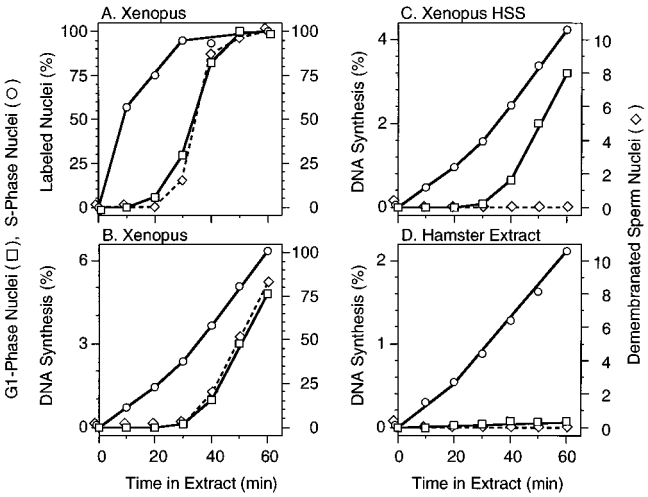

FIG. 3. Characteristics of DNA synthesis in G_1 nuclei in cell extract were consistent with initiation of DNA replication. Either G_1 nuclei, S-nuclei, or demembranated *Xenopus* sperm nuclei (note that the scale for sperm nuclei is on the rightmost *y* axis) were introduced into *Xenopus* egg extract supplemented with either 250 μM BrdUTP (A) or 25 μCi of [α-^{32}P]dATP (6,000 Ci/mmol; Amersham) per ml (B). At the indicated times, aliquots from these reaction mixtures were removed, and either the percentage of nuclei that incorporated BrdU (A) or the percentage of DNA substrate that replicated (B) was calculated. These same substrates were also incubated in *Xenopus* egg extract that had been cleared of insoluble components by high-speed centrifugation (HSS) (C) or in cytosolic extract from S-phase CHO cells (D) supplemented with [α-^{32}P]dATP, and the percentage of input DNA that replicated was determined. Similar results were obtained with Dounce nuclei and scraped nuclei.

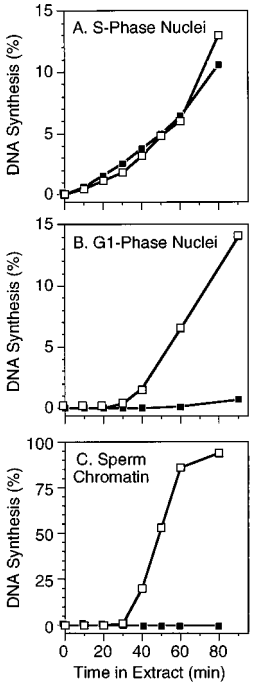

FIG. 4. 6-DMAP specifically inhibits initiation of DNA replication in G_1 nuclei. Either S nuclei (A), G1 nuclei (B), or demembranated *Xenopus* sperm nuclei (C) were introduced into extract from unactivated *Xenopus* eggs supplemented with [α-^{32}P]dATP in the presence (■) or absence (□) of 3 mM 6-DMAP. Extract was released into interphase with 0.3 mM $CaCl_2$ (6), and the percentage of input DNA that replicated was determined. Similar results were obtained with Dounce nuclei and scraped nuclei.

three substrates, but DNA synthesis in G_1 nuclei and sperm nuclei was delayed for 10 to 30 min, whereas DNA synthesis in S nuclei began immediately. This delay was observed both by determining the percentage of nuclei that incorporated BrdUTP into replication foci (Fig. 3A) and by measuring incorporation of [α-^{32}P]dATP into acid-precipitable material (Fig. 3B). With demembranated sperm nuclei, this delay may represent the time required for reassembly of a nuclear membrane (9); however, this explanation did not appear to apply to G_1 nuclei. First, G_1 nuclei were prepared by lysing cells in digitonin, a technique that leaves the nuclear membrane intact (2, 62). This was confirmed for the G_1 nuclei in these experiments by their ability to exclude 160-kDa immunoglobulin G and 70-kDa dextran (data not shown). Furthermore, a high-speed supernatant fraction of *Xenopus* egg extract (9) that was incapable of assembling nuclear membranes or replicating sperm DNA was still capable of initiating DNA synthesis in G_1 nuclei (Fig. 3C). Thus, the delay observed before DNA synthesis begins in G_1 nuclei must reflect the need for additional events required prior to formation of replication forks such as assembly of preinitiation complexes at sites where replication will occur.

The requirement for an additional event in G_1 nuclei that had already occurred in S nuclei was further demonstrated by the sensitivity of G_1 nuclei to 6-dimethylaminopurine (6-DMAP). Previous results have shown that metaphase-arrested *Xenopus* egg extract treated with either 6-DMAP (7) or staurosporine (60), two general inhibitors of protein kinase activities, and then activated by calcium to begin DNA synthesis is unable to support initiation of DNA synthesis in sperm nuclei but is able to support DNA synthesis at preexisting replication forks. We confirmed the inhibitory effect of 6-DMAP on initiation of DNA synthesis in sperm nuclei and extended it to initiation of DNA synthesis in G_1 nuclei (Fig. 4). In contrast, DNA synthesis in S nuclei was unaffected by 6-DMAP, suggesting that formation of preinitiation complexes required a protein kinase activity.

To confirm that nascent DNA strands synthesized in G_1 nuclei were initiated de novo in *Xenopus* egg extract, G_1 nuclei or S nuclei were incubated in *Xenopus* egg extract containing [α-^{32}P]dATP and BrdUTP, and the density of nascent [^{32}P] DNA chains produced after 10, 20, and 30 min of incubation was analyzed by centrifugation to equilibrium in alkaline Cs_2SO_4. Nascent [^{32}P]DNA strands that arise by extending preexisting DNA chains vary in density from light to heavy, depending on the relative lengths of the light portion (DNA synthesized in the hamster cell) and heavy portion (DNA synthesized in *Xenopus* egg extract in the presence of BrdUTP). On the other hand, [^{32}P]DNA strands that originate exclusively in *Xenopus* egg extract are completely substituted with BrdUTP and thus form a single heavy-strand species. As expected, [^{32}P]DNA appeared in S nuclei within the first 10 min of incubation in *Xenopus* egg extract and resulted primarily from extension of preexisting DNA chains. In contrast, ^{32}P-DNA appeared in G_1 nuclei only after 30 min of incubation and consisted predominantly of fully substituted DNA chains (Fig. 5). Taken together, these results demonstrate that *Xenopus* egg extract initiates DNA synthesis de novo in G_1 nuclei.

***Xenopus* egg extract initiates DNA synthesis at specific chromosomal sites in G_1 nuclei.** Previous studies have mapped an origin of DNA replication (ori-β) downstream of the DHFR gene in both CHOC 400 cells and its diploid single-copy parent, CHO cells. Most replication forks in this region appear to travel bidirectionally away from an OBR located within a 0.5- to 2-kb locus about 17 kb downstream of the DHFR gene, although initiation bubbles can be detected throughout a

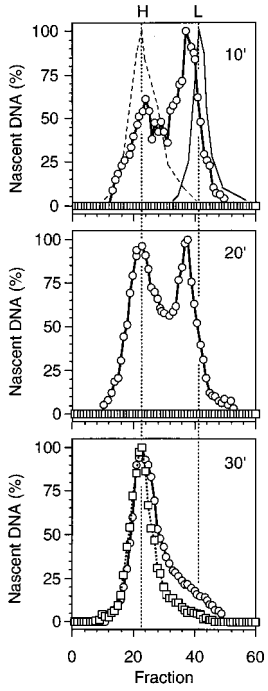

FIG. 5. DNA synthesis induced in G_1 nuclei resulted from de novo initiation of new DNA strands. Either G_1 nuclei (□) or S nuclei (○) from CHOC 400 cells were added to *Xenopus* egg extract supplemented with [α-^{32}P]dATP and BrdUTP. After 10, 20, or 30 min, genomic DNA was isolated and subjected to alkaline density gradient centrifugation in Cs_2SO_4 as in Fig. 1. Similar results were obtained with Dounce nuclei and scraped nuclei. H, heavy; L, light.

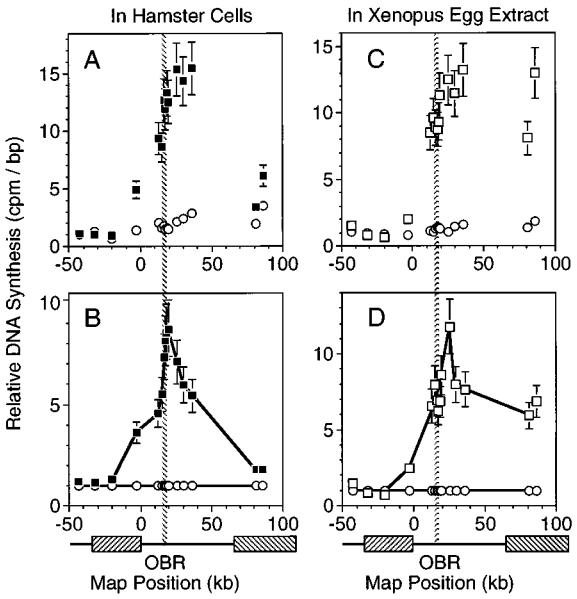

FIG. 7. DNA synthesis induced in G_1 nuclei was initiated at specific sites. (A and B) In hamster cells. (A) Nascent DNA in G_1/S nuclei (■) isolated from CHOC 400 cells was labeled with [α-^{32}P]dATP and then hybridized to the 15 double-stranded DNA probes described in Fig. 6. As a control, the same protocol was carried out with exponentially proliferating CHOC 400 cells (○). The amount of [^{32}P]DNA per base pair of probe relative to the mean value of probes H, I, and A (these three probes always bound the least [^{32}P]DNA from G_1/S nuclei) was determined directly by PhosphorImager analysis. (B) To normalize for any experimental variation between probes not specifically due to G_1/S synchrony, the relative value for each probe in panel A was divided by the corresponding value for exponentially proliferating nuclei. (C and D) In *Xenopus* egg extract. (C) G_1-phase CHOC 400 nuclei were incubated for 1 h in *Xenopus* egg extract containing aphidicolin. Nascent DNA was then labeled with [α-^{32}P]dATP and hybridized as in panel A (□). As a control, the same protocol was carried out with nuclei from exponentially proliferating CHOC 400 cells (○). (D) The data from panel C were normalized as described for panel B. The vertical line through each graph shows the position of the previously mapped OBR, and the horizontal axis shows the relative map positions of the probes and transcription units as they are shown in Fig. 6. Shown are the mean values from three independent experiments and the standard errors of the means.

larger initiation zone (Fig. 6) (14, 26, 30, 31, 38, 88). Additional initiation sites have recently been detected within the 2BE2121 gene, although not within the DHFR gene (31), and evidence exists for another OBR at ori-γ (46). Therefore, the ability of *Xenopus* egg extract to initiate replication at specific genomic loci in digitonin nuclei was evaluated quantitatively and extensively in digitonin nuclei by pulse-labeling the earliest-replicated DNA and then hybridizing it to 15 unique probes distributed over a 130-kb region that included the DHFR ori-β (early labeled fragment hybridization method; Fig. 6). These probes were selected because they annealed to a unique DNA fragment of the predicted size in CHOC 400 genomic DNA digested with either *Xba*I or *Eco*RI (data not shown).

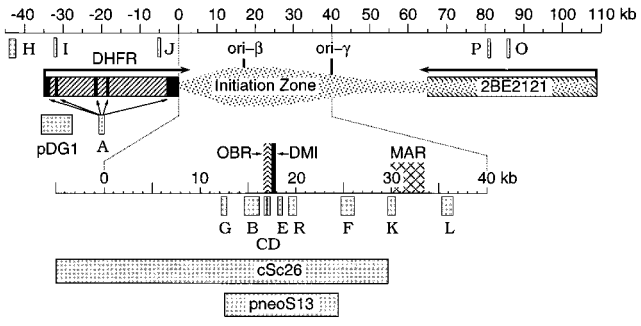

FIG. 6. Locations of unique DNA probes in the DHFR gene region. The approximate positions of the 15 probes used to map the sites of initiation of chromosomal DNA replication, as well as plasmid and cosmid DNA inserts used in Fig. 9 to 11, are shown in relationship to the DHFR and 2BE2121 transcription units (direction of transcription is indicated with an arrow). Also shown are the positions of ori-β and ori-γ and of the initiation zone mapped by 2D gel electrophoresis (30, 31). The ori-β region is expanded to show the positions of the OBR, a matrix-associated region (MAR) (29), and a densely methylated island (DMI) (89). Probe A contains exons 1 to 5 (totaling 488 nt) and 155 nt of exon 6 from the DHFR cDNA and is positioned at the center of the DHFR gene to represent its average position. Locations of hamster DNA contained in pneoS13, pDG1, and cSc26 are indicated.

The efficacy of this origin mapping protocol was demonstrated by using CHOC 400 cells that had initiated DNA replication in vivo. Cells synchronized by mitotic selection were allowed to proceed into S phase in the presence of aphidicolin, a specific inhibitor of replicative DNA polymerases (92), in order to accumulate cells at their G_1/S boundary with newly formed replication forks arrested close to their sites of initiation (48). Nuclei prepared from these G_1/S-phase cells were briefly incubated with [α-^{32}P]dATP before purifying nascent DNA and hybridizing it simultaneously to each of the 15 probes shown in Fig. 6. The amount of [^{32}P]DNA that annealed was quantified directly with a PhosphorImager. As expected, most of the hamster nascent [^{32}P]DNA from this genomic region hybridized predominantly to the ori-β locus, consistent with bidirectional DNA replication from this replication origin (Fig. 7A). In contrast, nascent [^{32}P]DNA from exponentially proliferating CHOC 400 cells, labeled identically to nascent DNA from G_1/S-phase cells, hybridized almost equally well to each of the probes (Fig. 7A), consistent with a population of cells containing replication forks distributed ran-

domly throughout the genome. To normalize for any experimental variation among the various probes that is not due specifically to the synchrony of cells at the onset of S phase, the ratio of [^{32}P]DNA per base pair of probe obtained with G_1/S-phase cells to [^{32}P]DNA per base pair of probe obtained with exponentially proliferating cells was calculated. These results (Fig. 7B) revealed that CHOC 400 cells initiated DNA synthesis at or near the same OBR that had been previously mapped downstream of the DHFR gene in CHO cells.

To determine whether *Xenopus* egg replication factors initiated DNA synthesis specifically at the same genomic locus chosen by hamster cells, G_1 nuclei were incubated in *Xenopus* egg extract for 1 h in the presence of aphidicolin. Nuclei were then washed free of aphidicolin, nascent DNA chains were labeled briefly with [α-^{32}P]dATP, and the resulting [^{32}P]DNA chains were annealed simultaneously to each of the 15 DNA probes. As with nuclei that initiated DNA replication in hamster cells, most of the [^{32}P]DNA synthesized in G_1 nuclei originated near the OBR (Fig. 7C). These data were then normalized to those obtained with nuclei isolated from exponentially growing cells that were incubated in *Xenopus* egg extract in an identical manner. These results (Fig. 7D) revealed that nascent [^{32}P]DNA originated at or near the same sites utilized by hamster cells. Similar results were obtained with G_1 nuclei that were incubated in *Xenopus* egg extract for up to 3 h in the presence of aphidicolin (see Fig. 12) or up to 45 min without aphidicolin.

The ability of *Xenopus* egg extract to initiate preferentially at DHFR ori-β was confirmed by mapping the OBR with the Okazaki fragment distribution method. If replication forks are initiated bidirectionally at or close to the same site in each cell, then Okazaki fragments prepared from these cells will hybridize preferentially to the DNA strand that serves as template for discontinuous DNA synthesis. Thus, an OBR can be identified by the transition from continuous to discontinuous DNA synthesis that will occur on each DNA strand (15, 20, 88). Therefore, to locate the OBR used in CHOC 400 cells, G_1/S nuclei were incubated briefly with [α-^{32}P]dATP, and Okazaki fragments were then isolated and hybridized to individual strands of the DHFR DNA probes. The fraction of ^{32}P-labeled Okazaki fragments that annealed to the 3′-to-5′ template (template polarity oriented as in Fig. 6) revealed that in G_1/S cells, most replication forks originated at an OBR 15 to 20 kb downstream of the DHFR gene (Fig. 8A), in general agreement with previously published reports of studies using CHO cells (15, 88). As a control, genomic DNA from CHOC 400 cells was radiolabeled in vitro by using random primers and hybridized to these same probes. This DNA hybridized equally to both strands (Fig. 8A). When the same experiment was carried out with G_1 nuclei incubated in *Xenopus* egg extract for 45 min, the distribution of Okazaki fragments between the two templates and the location of a transition from continuous to discontinuous DNA synthesis were found to be similar to those in G_1/S nuclei that had initiated DNA replication in hamster cells (Fig. 8B). Therefore, *Xenopus* egg extract preferred to initiate at or close to ori-β.

Burhans et al. (15) reported that an OBR could be mapped in exponentially growing CHOC 400 cells by the Okazaki fragment distribution method. In trying to reproduce this experiment, we discovered that these cells had routinely been split from confluency 12 to 14 h prior to analysis (13). FACS analysis showed that under these conditions, CHOC 400 cells were actually synchronized in early S phase (data not shown). We concluded that strong biases in Okazaki fragment hybridization to separated DNA templates required cells that be synchronized at their G_1/S-phase border (data not shown). This

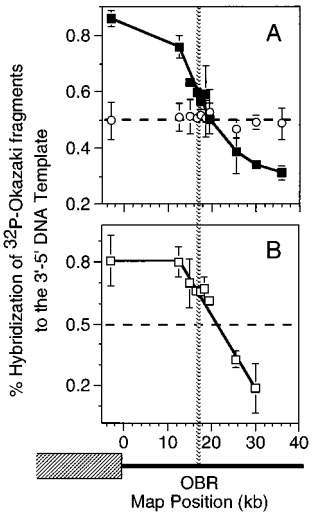

FIG. 8. DNA synthesis induced in G_1 nuclei resulted from replication forks that emanated from specific sites. (A) Nascent DNA in G_1/S nuclei (■) was labeled with [α-^{32}P]dATP, and Okazaki fragments were isolated and then hybridized to single-stranded probes J through L in Fig. 6. As a control, CHOC 400 genomic DNA was radioactively labeled with random primers (Stratagene Prime It Kit) and then hybridized to duplicate samples of these DNA probe preparations (○). (B) G_1 nuclei (□) were incubated in *Xenopus* egg extract for 45 min. Okazaki fragments were then labeled, isolated, and hybridized to single-stranded DNA probes as in panel A. For each experiment, two independent preparations of single-stranded DNA were hybridized, and the mean values are presented.

would be expected if only a fraction of the amplified ori-β loci initiate replication in each cell division cycle. In unsynchronized cells, passively replicated amplicons could be replicated by forks moving in either direction, thus reducing significantly the bias produced by initiation at a specific OBR (e.g., see Fig. 2 in reference 15).

Site-specific initiation of replication is not observed with naked DNA. To determine whether the DHFR initiation locus constituted a preferred site for DNA replication in *Xenopus* egg extract even in the absence of a CHO nuclear structure, the relative replication efficiencies of three plasmids were compared. pneoS13 contained an 11.9-kb CHO DNA fragment encompassing the DHFR ori-β OBR (Fig. 6), pDG1 contained a 7.5-kb DNA fragment from the DHFR structural gene that lies outside the ori-β initiation zone (Fig. 6), and pDGλ8.6 contained an 8.6-kb segment of bacteriophage λ DNA. An equimolar mixture of these three plasmids was incubated in *Xenopus* egg extract, and the percentage of molecules that had undergone at least one complete round of semiconservative replication was determined by conversion of monomer-length plasmid DNA from *Dpn*I sensitive to *Dpn*I resistant (see Materials and Methods). All three plasmids replicated efficiently and to the same extent, regardless of whether plasmid DNA was introduced as circular monomers or long linear concatemers (Fig. 9). The same experiments were carried out in vivo by injecting 1.6 ng of each plasmid into unactivated *Xenopus* eggs and then allowing 1 h for the DNA to assemble into chromatin before activating the eggs with calcium in order to initiate DNA replication as previously shown for pneoS13 (43). Again, all three plasmids replicated efficiently and to the same extent (data for pneoS13 shown in reference 43). To determine whether a preference for ori-β-containing plasmids became apparent only after two or more rounds of DNA replication in vivo, DNA replication products were digested with *Mbo*I instead of *Dpn*I, and the conversion from *Mbo*I resistant to *Mbo*I sensitive was monitored. *Mbo*I cuts the same site as *Dpn*I, but

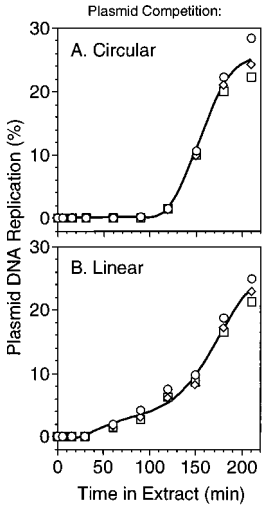

FIG. 9. *Xenopus* egg extract did not preferentially initiate replication within a plasmid containing ori-β. Plasmids pneoS13 (○), pDG1 (□), and pDGλ8.6 (◇) were mixed in equimolar quantities and incubated in *Xenopus* egg extract. At the indicated times, aliquots of this reaction mixture were removed, and the percentage of each plasmid type that replicated completely was calculated as described in Materials and Methods. Plasmid DNA was present either as circular monomers (A) or linear concatemers (B).

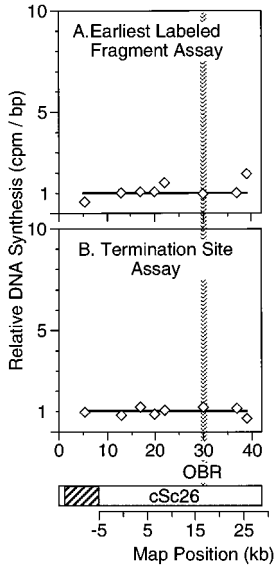

FIG. 10. *Xenopus* egg extract did not recognize ori-β as a specific initiation site. (A) Earliest labeled fragment assay. Cosmid cSc26 (Fig. 6) was incubated for 2 h in *Xenopus* egg extract (10 ng/μl) supplemented with 100 μg of aphidicolin per ml. This allowed naked DNA to be assembled into pseudonuclei (77) and initiate replication while simultaneously preventing replication forks from traveling away from their sites of origin. Pseudonuclei were then washed free of the aphidicolin and incubated in fresh *Xenopus* egg extract containing [α-^{32}P]dATP (25 μCi/ml) for 1 min to label nascent DNA. On the basis of the rate of fork movement in *Xenopus* egg extract (67), less than 3% of the 42-kb cosmid should have replicated under these conditions. An excess of unlabeled dATP (5 mM plus 5 mM $MgCl_2$) was then added, and the reaction continued for an additional 20 min to complete replication. DNA was isolated, digested with *Eco*RI and *Dpn*I, and fractionated by electrophoresis in an 0.8% agarose gel, and the amount of radioactivity in each *Dpn*I-resistant *Eco*RI DNA fragment was determined by PhosphorImager analysis. To control for variation in base composition and fragment size, the relative counts per minute per fragment was normalized to the value for cSc26 DNA that had been radiolabeled uniformly for 3 h in *Xenopus* egg extract. Each point on the graph is positioned at the center of an *Eco*RI fragment and plotted a scale similar to that in Fig. 7 to facilitate comparison. The stippled box represents cosmid vector sequences (pHC79), and the open box represents hamster DNA. The vertical line through the graph denotes the position of the OBR. (B) Termination site assay. cSc26 was incubated in *Xenopus* egg extract for 105 min to achieve the maximum rate of DNA replication. Nascent DNA was then labeled for 5 min by addition of [α-^{32}P]dATP. DNA was extracted and passed twice through a benzoylnaphthol DEAE-cellulose column in order to remove any partially replicated molecules that contained single-stranded regions at their replication forks (37). Fully replicated [^{32}P]DNA was digested with *Eco*RI and *Dpn*I and fractionated by gel electrophoresis, and the amount of [^{32}P]DNA per *Eco*RI fragment was determined as in panel A.

only when the site is completely unmethylated (27, 43). Again, no difference was detected between the replication efficiencies of the three plasmids (data for pneoS13 shown in reference 43). These results demonstrate that plasmids containing the DHFR ori-β region are not a preferred substrate for initiation of replication in either *Xenopus* eggs or egg extract, consistent with previous reports that *Xenopus* eggs initiate replication efficiently in virtually any bare DNA molecule (22).

Nevertheless, the possibility remained that *Xenopus* eggs preferentially initiate replication at ori-β within a given stretch of DNA. Therefore, a cosmid containing most of the DHFR ori-β initiation zone (cSc26 [Fig. 6]) was incubated in *Xenopus* egg extract, and replication site specificity was analyzed by two independent methods. First, the same earliest labeled fragment assay that revealed the ability of *Xenopus* egg extract to induce site-specific initiation of replication in hamster G_1 nuclei (Fig. 7) was modified and carried out with cSc26 (Fig. 10). None of the eight *Eco*RI restriction fragments was labeled preferentially (Fig. 10A), suggesting that *Xenopus* egg extract does not initiate replication at specific sites when bare DNA is provided as the substrate. To confirm this conclusion, the termination site for DNA replication was also mapped. Circular DNA molecules that initiate bidirectional replication at a unique site will generally terminate replication 180° from the origin, regardless of the DNA sequence at the termination site (93). Therefore, cosmid DNA was briefly labeled in *Xenopus* egg extract, and those molecules that had completed replication during this brief period were purified and digested with *Eco*RI, and the amount of radiolabel per nucleotide was determined for each fragment. None of the eight fragments was labeled preferentially (Fig. 10B), again suggesting that replication did not begin at a specific site within the cosmid DNA.

Finally, we considered the possibility that the ability to recognize specific replication origins does not occur until later in *Xenopus* development. For example, replication appears to initiate within the nontranscribed spacer regions of rRNA genes in *Xenopus* differentiated tissue (11) but at random sites throughout the rRNA gene repeats in *Xenopus* embryonic tissue (50). Therefore, fertilized *Xenopus* eggs were injected with an equimolar mixture of all three plasmids in the form of either circular monomer or linear concatemer DNA. *Xenopus* embryos were collected at various times after fertilization up to the free swimming tadpole stage, and the amount of *Dpn*I-resistant plasmid DNA per embryo was determined for each plasmid. All three plasmids replicated efficiently, exponentially, and at equivalent rates up to the blastula stage, at which point all three plasmids ceased to replicate (Fig. 11). Thus, following the mid-blastula transition (MBT), *Xenopus* differentiated tissues replicate poorly, if at all, exogenous DNA sequences that were introduced as bare DNA, regardless of the presence or absence of ori-β.

Site-specific initiation of replication requires an intact nucleus. To determine whether an intact differentiated nuclear structure was required for site-specific initiation of replication, nuclei were isolated from G_1-phase CHOC 400 cells by four different methods. Nuclei prepared under isotonic conditions

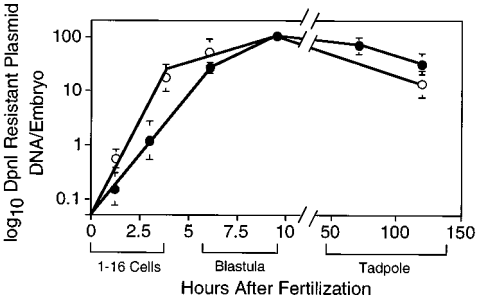

FIG. 11. Efficient and promiscuous replication of plasmid DNA ceases near the MBT. Plasmids pneoS13, pDG1, and pDGλ8.6 in the form of either circular monomers (○) or linear concatemers (●) were mixed in equimolar quantities, and 5 ng of this mix was coinjected into fertilized *Xenopus* eggs as described previously (47). DNA was isolated (43) from 10 embryos at the indicated times, and the relative amounts of *Dpn*I-resistant plasmid DNA per embryo was determined. Developmental stages observed at each time interval are indicated.

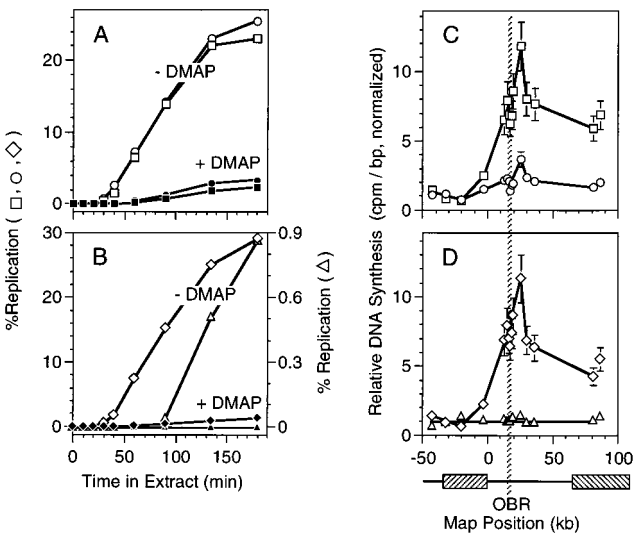

FIG. 12. Site-specific initiation of DNA replication depended on the method of nucleus preparation. Nuclei were prepared from G_1-phase CHOC 400 cells by four different methods (see Materials and Methods): digitonin nuclei (□), Dounce nuclei (○), scraped nuclei (◇), and Triton nuclei (△). (A and B) Each of these substrates was introduced into an unactivated *Xenopus* egg extract supplemented with [α-^{32}P]dATP in the presence (closed symbols) or absence (open symbols) of 6-DMAP, and the percentage of DNA substrate that replicated was determined as in Fig. 4. (C and D) G_1 nuclei were incubated in interphase *Xenopus* egg extract supplemented with 100 μg of aphidicolin per as in Fig. 7 except that incubation was for 3 h at 21°C. Nascent DNA was radiolabeled and hybridized to the 15 probes illustrated in Fig. 6 as described in the legend to Fig. 7. The mean values from three independent experiments were plotted along with the standard errors of the means.

either by lysing cells in digitonin (2) (digitonin nuclei) or by scraping cells from their culture dish (70) (scraped nuclei) remained impermeable to fluorescein-labeled immunoglobulin G or 70-kDa dextran sulfate and were therefore considered to retain an intact nuclear membrane (2, 62). In contrast, nuclei prepared in hypotonic buffer by mechanically lysing cells in a Dounce homogenizer (48) (Dounce nuclei) were swollen and permeable to these same molecules. Finally, since previous results suggested that *Xenopus* egg extract can reprogram replication in *Drosophila* polytene nuclei prepared with a nonionic detergent (84), some of the Dounce nuclei were washed in Triton X-100 (Triton nuclei).

The characteristics of DNA synthesis in scraped nuclei, Dounce nuclei, and Triton nuclei were similar to those described for digitonin nuclei in Fig. 1 through 5 except that Triton nuclei underwent a longer delay (90 min) before DNA synthesis was detected (Fig. 12B). Since elongation of preexisting replication forks in S-phase Triton nuclei was also substantially reduced during the first 60 min in *Xenopus* egg extract (data not shown), nonionic detergents affected more than just the initiation process. The detergent effect was not the result of nuclear membrane permeabilization alone, because Dounce nuclei were indistinguishable from digitonin or scraped nuclei (Fig. 12A).

The ability of *Xenopus* egg extract to initiate DNA synthesis preferentially at ori-β was evaluated in all four G_1 nucleus preparations. In each case, initiation of DNA synthesis was inhibited by 6-DMAP (Fig. 12A and B), consistent with de novo initiation of DNA replication. However, when the early labeled fragment hybridization method previously used to map initiation sites in the DHFR gene region of digitonin nuclei (Fig. 7) was applied to other nucleus preparations, only the scraped nuclei initiated with a pattern identical to that of digitonin nuclei (Fig. 12C). In contrast, site specificity in Dounce nuclei was significantly reduced, while Triton nuclei failed to exhibit site specificity (Fig. 12D). Thus, site specific initiation of DNA replication in *Xenopus* egg extract required an intact nuclear structure.

DISCUSSION

A role for nuclear structure in establishing origins of DNA replication. Results presented here demonstrate that *Xenopus* egg extract, a system previously observed to initiate DNA replication only randomly throughout DNA molecules, can initiate replication at specific sites when the DNA is presented in the form of nuclei isolated from G_1-phase mammalian fibroblasts. DNA synthesis under these conditions resulted from replication and not repair of DNA, because it was semiconservative (Fig. 1) and involved the repeated synthesis and joining of Okazaki fragments to long nascent DNA strands (Fig. 2) on one arm of replication forks (Fig. 8). In addition, it was sensitive to inhibitors of replicative DNA polymerases and occurred at discrete foci within the nucleus.

DNA synthesis in G_1 nuclei differed from DNA synthesis in S nuclei in five ways. Taken together, these results demonstrated that DNA replication in G_1 nuclei resulted from de novo initiation of replication rather than continuation of DNA synthesis at preformed replication forks. First, all of the S nuclei carried out DNA synthesis in extracts from either S-phase hamster cells or *Xenopus* eggs, whereas all of the G_1 nuclei carried out DNA synthesis only in extracts from *Xenopus* eggs (Fig. 3). Therefore, G_1 nuclei required an additional factor that was abundant in *Xenopus* eggs but not in hamster extracts. Second, DNA synthesis in G_1 nuclei, but not in S nuclei, was delayed (Fig. 3), suggesting that G_1 nuclei undergo an additional event that had already occurred in S nuclei. Third, DNA synthesis in G_1 nuclei, but not in S nuclei, was inhibited by 6-DMAP (Fig. 4), suggesting that initiation of DNA replication requires a protein kinase activity. Fourth, nascent DNA chains were completely labeled in G_1 nuclei but not in S nuclei (Fig. 5), consistent with de novo initiation of DNA synthesis in G_1 nuclei and continuation of DNA synthesis at preinitiated sites in S nuclei. Finally, except for a requirement to assemble nuclear membrane in low-speed extract, the characteristics of DNA synthesis in G_1 nuclei were similar to those in *Xenopus* sperm chromatin (Fig. 3 and 4), a postmeiotic DNA substrate that had never entered S phase and therefore is devoid of replication forks.

The conclusion that initiation of DNA replication in G_1 nuclei occurred at specific DNA sites rather than at randomly chosen sites distributed throughout the genome was based on three methods for mapping replication origins. Preliminary studies identified early labeled DNA fragments in the DHFR gene region by briefly incubating nuclei isolated from CHOC 400 cells with [α-^{32}P]dATP, digesting their DNA with *Eco*RI, fractionating it by gel electrophoresis, and finally detecting [^{32}P]DNA by autoradiography. Results from these experiments (42) suggested that *Xenopus* egg extract continues DNA synthesis in S nuclei at sites that had been initiated in vivo (e.g., ori-β) and initiates DNA synthesis in G_1 nuclei at sites normally chosen by hamster cells (e.g., ori-β). However, these experiments were not quantifiable, and results with G_1 nuclei were difficult to reproduce because they were done with Dounce nuclei that we later discovered gave variable results. Therefore, a second method using quantitative hybridization of newly synthesized DNA to double-stranded DNA probes was used to reveal the genomic locations of newly labeled nascent DNA (Fig. 7 and 12). The results obtained with this method were confirmed by quantitative hybridization of Okazaki fragments to single-stranded DNA probes to reveal the transition between continuous and discontinuous DNA synthesis on each template that defines an OBR (Fig. 8).

Site-specific initiation of DNA replication was observed in *Xenopus* egg extract only when DNA was presented as an intact nuclear structure that had been assembled in vivo (digitonin or scraped nuclei). If circular or linear plasmid DNA containing the DHFR ori-β region was assembled into a nuclear structure by *Xenopus* egg extract (Fig. 9 and 10) or if damaged nuclei were incubated in the extract (Fig. 12, Dounce or Triton nuclei), site specificity was lost or greatly reduced.

Initiation of DNA replication in hamster nuclei by *Xenopus* egg extract was confined to the same initiation zone recognized by hamster cells, occurring primarily at or close to the same sites preferred by hamster cells (Fig. 7 and 8). The only difference was that *Xenopus* egg extract appeared to initiate DNA replication at sites located further downstream of the DHFR gene more frequently than this occurred in hamster cells. The presence of additional initiation sites detected by *Xenopus* egg replication factors is consistent with reports of at least one additional OBR in this region (ori-γ [46]; Fig. 6) and the presence of weak initiation sites extending into the 2BE2121 gene in CHOC 400 cells (Fig. 6 and reference 31). Therefore, although the relative preference for initiation sites within the DHFR locus may be slightly altered in *Xenopus* egg extract, initiation is confined to the same set of potential initiation sites utilized normally by CHOC 400 cells and the preferred site of initiation is consistently at or close to ori-β.

How specific is the site selection process at the DHFR locus? Precise interpretation of the origin mapping data in Fig. 7 depends on the efficacy of aphidicolin to arrest replication forks and the rates of fork movement throughout this region during the time of radiolabeling. Aphidicolin does not prevent synthesis of short RNA-primed nascent DNA chains (76) and therefore allows both simian virus 40 (24) and cellular (42) chromosomes to initiate DNA replication by forming replication forks but limits subsequent DNA synthesis according to the dose and time of exposure. In fact, we found that CHOC 400 cells arrested with 10 μg of aphidicolin per ml still incorporated enough BrdU in a 30-min labeling time to stain positive with anti-BrdU antibodies (data not shown). Therefore, the simplest interpretation is that most initiation events in G_1/S-phase CHOC 400 cells occurred at or near the OBR previously mapped in CHO cells, resulting in a gradient of replication forks arrested at various distances from the OBR in both directions.

Nonetheless, the transition between continuous and discontinuous DNA synthesis that identifies the OBR in the amplified DHFR ori-β locus in CHOC 400 cells was approximately 2 kb further downstream (Fig. 8) and appeared to occur over a broader region of DNA (Fig. 7 and 8) than previously reported in the unamplified DHFR locus of CHO cells (15, 88). Burhans et al. (15) reported that the OBR was at the same site in CHOC 400 cells as in CHO cells, but the analysis in CHOC 400 cells was limited primarily to probes F and G (15, 16), and these data are consistent with those presented here. We suggest that differences observed between initiation events in CHO and CHOC 400 cells reflect differences in nuclear structure between cells containing one copy of the DHFR ori-β locus per haploid genome (CHO cells) and cells containing ~500 tandem copies per haploid genome (CHOC 400 cells). In support of this hypothesis, the amplified cluster of DHFR genes has been observed to bulge out of CHOC 400 nuclei (90), and this altered nuclear structure may affect selection of initiation sites in some or all of the amplified repeats.

An emerging picture of replication origins in metazoan chromosomes. The results summarized above and in the introduction suggest a simple paradigm (Fig. 13) for mammalian origins that could account for why replication origins are difficult to identify in transient assays with plasmid DNA incubated in *Xenopus* eggs or egg extracts or transfected into mammalian cells. Naked DNA contains many potential sites where replication can begin. These sites most likely correspond to easily unwound DNA sequences that are components of replication origins in simple (75) as well as metazoan (33) genomes and that can promote site-specific replication in plasmid DNA (51). When naked DNA is introduced into *Xenopus* eggs or egg extract, chromatin is assembled in the absence of histone H1, which is required for compaction of DNA into 30-nm fibers (94), and in the presence of only one nuclear lamin protein, a component of nuclear structure required for DNA replication (53, 78), instead of the three found in differentiated cells (5, 86). The resulting nuclear structure is less compact than a typical nucleus in differentiated cells (72). In addition, DNA transcription, which can suppress DNA replication (45), is practically absent in *Xenopus* eggs until the MBT (81). This relaxed environment may allow replication to initiate at any of the many potential origins located within a large DNA region, thus facilitating rapid replication of the *Xenopus* embryonic genome. However, at the MBT, changes in chromatin and nuclear structure as well as the onset of transcription may repress initiation of DNA replication at many potential initiation sites while facilitating it at others, resulting in site-specific initiation of replication in nuclei of differentiated cells (the Jesuit model [14, 25, 26]). This would reduce the number of initiation sites in differentiated cells relative to early embryonic cells, thereby making it easy to identify specific initiation sites in differentiated cells but not in rapidly dividing embryonic cells (10, 12, 69, 82). Fewer replication origins would contribute to the increased length of the cell division cycle that occurs at the MBT (56). Moreover, it would make replication of injected DNA more difficult, as observed when injected DNA reaches the blastula stage (Fig. 11).

Sites favored as replication origins in differentiated cells may consist of an easily unwound DNA sequence associated with another origin component such as a densely methylated island (89) or an origin recognition element (25, 65). Moreover, the ability of soluble factors in a cell-free system to detect site-specific replication only in preformed nuclei suggests that replication origins are composed of specific DNA sequences or-

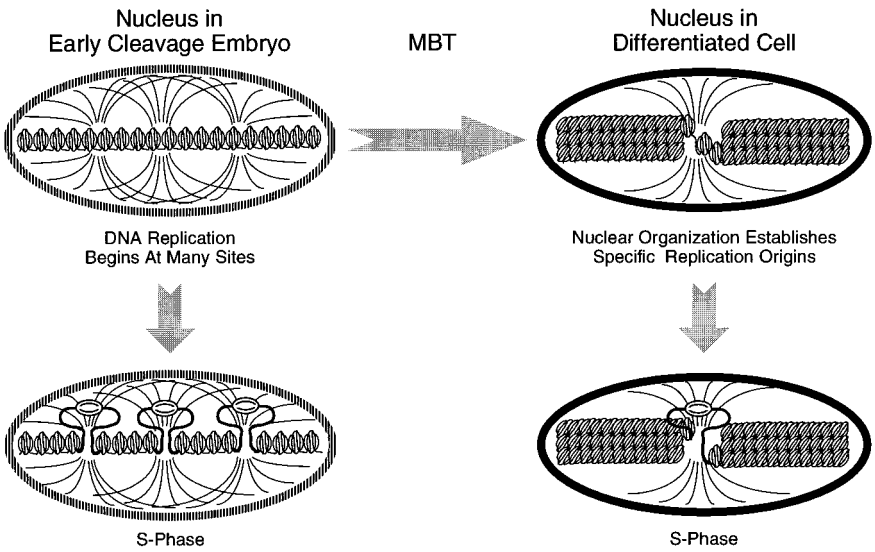

FIG. 13. Nuclear structure restricts initiation to specific sites. We propose that *Xenopus* eggs assemble DNA into a relaxed nuclear structure that permits initiation of DNA replication within many sequences that are easily unwound, allowing the early-cleavage-stage amphibian embryo to rapidly replicate its entire genome. During the MBT, changes in chromatin and nuclear structure restrict the number of sites that can be used as origins of replication (see text). The time required for cell division begins to increase at the MBT (56), consistent with a reduction in the number of replication sites in differentiated cells. The MBT marks the onset of zygotic gene transcription (56) and cell differentiation (72) in *Xenopus* embryos and is accompanied by changes that could repress initiation at some sites while facilitating initiation at others. For example, histone H1 appears and condenses chromatin into a 30-nm fiber (94), and this is shown as a more compacted structure in the figure. The composition of nuclear lamin proteins is altered (5, 86), the distribution of proliferating cell nuclear antigen changes (61), and nuclear volume is reduced (72). The curved lines in the figure represent presumptive attachments to fixed components of the nucleus such as the nuclear matrix. In the early-cleavage-stage embryo, replicons are shown to be initiated at many possible sites but at regular intervals as predicted by Hyrien and Méchali (50).

ganized into a nucleoprotein complex that includes some component of nuclear structure, such as matrix attachment regions (Fig. 13). Those DNA sequences that determine which chromosomal locations will be selected as OBRs comprise a heritable replication origin whose genetically required elements may be revealed only within the context of a cellular chromosome organized into a nucleus, a requirement that may be difficult to fulfill with small plasmid DNA molecules (17).

The fact that *Xenopus* egg extract can initiate DNA replication at specific sites within nuclei isolated from G_1-phase hamster fibroblasts demonstrates that replication origins in differentiated mammalian cells are established several hours prior to the onset of S phase. Since DNA replication takes place within a supramolecular complex that is attached to the nuclear matrix (49), some components of this complex may be assembled in advance of DNA synthesis to establish the sites where replication eventually initiates. This would be analogous to the prereplicative nucleoprotein complex identified in *Saccharomyces cerevisiae* that associates with origins at the beginning of G_1 phase (28). One of these components may be replication protein A, an essential replication enzyme that binds to chromatin prior to a protein kinase-dependent step necessary for the initiation of DNA synthesis (1, 34). Soluble factors in the *Xenopus* egg cytosol may activate preinitiation complexes in G_1 nuclei during the 10- to 30-min delay before DNA synthesis begins in G_1 nuclei (Fig. 3 to 5). In sperm chromatin, this delay is required for nuclear membrane assembly (9), but digitonin and scraped nuclei retain an intact nuclear membrane, and *Xenopus* egg extracts that were depleted of their membrane-assembling capacity (sperm chromatin was inactive) were still able to efficiently initiate DNA synthesis in G_1 nuclei (Fig. 3).

One hallmark of DNA replication in cellular chromosomes that distinguishes it from replication in animal viruses such as simian virus 40 (25) and bovine papillomavirus (40) is that initiation is restricted to once per origin per S phase. If a replication origin consists of a nucleoprotein complex rather than simply a DNA sequence, then activation of such an origin would be limited to once per S phase simply as a result of its disruption during the act of replication. For example, the prereplicative nucleoprotein complex in *S. cerevisiae* dissociates when replication begins (28). This would prevent reinitiation of replication, even in the continued presence of soluble initiation factors, until such time as the origin complex could be reassembled, perhaps through the participation of licensing factor at mitosis (22).

Role of *Xenopus* egg factor(s) in initiation of DNA replication in intact nuclei. Three characteristics of DNA synthesis in hamster nuclei suggested that it was dependent, at least in part, on *Xenopus* enzymes. First, DNA synthesis was more efficient at 21°C in *Xenopus* egg extract than at 37°C in S-phase hamster cell extract (Fig. 3). Second, the half-life of Okazaki fragments synthesized in G_1 nuclei replicating in *Xenopus* egg extract (20 s at 12°C) was characteristic of *Xenopus* sperm DNA replication in *Xenopus* egg extract (8) rather than DNA replication in mammalian cells (90 s at 26°C; Fig. 2). Finally, DNA synthesis occurred efficiently in *Xenopus* egg extract from 12 to 16°C and was arrested at temperatures below 10°C, whereas DNA synthesis in hamster cell extract occurred efficiently from 32 to 37°C and was arrested at temperatures below 22°C.

One of the *Xenopus* components required for initiation of DNA replication in intact G_1 nuclei is a protein kinase, because the protein kinase inhibitor 6-DMAP inhibited DNA synthesis in G_1 nuclei but had little or no effect on DNA synthesis in S nuclei (Fig. 4). Previous studies suggested that protein kinase inhibitors prevent the activity of a licensing factor that is required to initiate DNA replication in sperm nuclei incubated in *Xenopus* egg extract (7, 60). However, the 6-DMAP-sensitive step observed during initiation of DNA replication in G_1 nuclei is inconsistent with activation by licensing factor, because licensing factor cannot enter intact nuclei;

nuclei must first be permeabilized by either chemical agents or mitosis. Digitonin nuclei and scraped nuclei are impermeable to licensing factor activity (62). Therefore, the soluble, 6-DMAP-sensitive *Xenopus* factor(s) required for initiation of site-specific replication in mammalian nuclei with intact membranes does not include licensing factor. However, it is possible that licensing factor plays a role in changing the distribution of initiation events in nuclei with damaged membranes (Dounce nuclei and Triton nuclei).

In summary, we have shown that some component of nuclear structure plays a role in establishing origins of replication in mammalian cells and that this component is present several hours prior to S phase. The development of a cell-free system that can initiate DNA replication site specifically provides the opportunity to investigate the nature of this structure.

ACKNOWLEDGMENTS

We are indebted to Joyce Hamlin, Peter Dijkwel, and Howard Cedar for providing cloned segments from the DHFR locus; to William Burhans for helpful advice; and to Jia-Rui Wu and Patricia Kane for critical reading of the manuscript.

H.M. was supported in part by a fellowship from RIKEN.

REFERENCES

1. **Adachi, Y., and U. K. Laemmli.** 1994. Study of the cell cycle-dependent assembly of the DNA pre-replication centres in *Xenopus* egg extract. EMBO J. **13:**4153–4164.
2. **Adam, S. A., R. S. Marr, and L. Gerace.** 1990. Nuclear protein import in permeabilized mammalian cells requires soluble cytoplasmic factors. J. Cell Biol. **111:**807–816.
3. **Anachkova, B., and J. L. Hamlin.** 1989. Replication in the amplified dihydrofolate reductase domain in Chinese hamster ovary cells may initiate at two sites, one of which is a repetitive sequence element. Mol. Cell. Biol. **9:**532–540.
4. **Ariizumi, K., Z. Wang, and P. W. Tucker.** 1993. Immunoglobulin heavy chain enhancer is located near or in an initiation zone of chromosomal DNA replication. Proc. Natl. Acad. Sci. USA **90:**3695–3699.
5. **Benavante, R., G. Krohne, and W. W. Franke.** 1985. Cell type-specific expression of nuclear lamina proteins during development of *Xenopus laevis*. Cell **41:**177–190.
6. **Berberich, S., A. Trivedi, D. C. Daniel, E. M. Johnson, and M. Leffak.** In vitro replication of plasmids containing human c-myc DNA. J. Mol. Biol., in press.
7. **Blow, J. J.** 1993. Preventing re-replication of DNA in a single cell cycle: evidence for a replication licensing factor. J. Cell Biol. **122:**993–1002.
8. **Blow, J. J., and R. A. Laskey.** 1986. Initiation of DNA replication in nuclei and purified DNA by a cell-free extract of *Xenopus* eggs. Cell **47:**577–587.
9. **Blow, J. J., and A. M. Sleeman.** 1990. Replication of purified DNA in *Xenopus* egg extract is dependent on nuclear assembly. J. Cell Sci. **95:**383–391.
10. **Blumenthal, A. B., H. J. Kreigstein, and D. S. Hogness.** 1973. The units of DNA replication in *Drosophila melanogaster* chromosomes. Cold Spring Harbor Symp. Quant. Biol. **38:**205–223.
11. **Bozzoni, I., C. T. Balarki, F. Amaldi, and M. Buorngiorno-Nardelli.** 1981. Replication of ribosomal DNA in *Xenopus laevis*. Eur. J. Biochem. **118:**585–590.
12. **Buongiorno-Nardelli, M., G. Micheli, M. T. Carri, and M. Marilley.** 1982. A relationship between replicon size and supercoiled loop domains in the eukaryotic genome. Nature (London) **298:**100–102.
13. **Burhans, W. C. (Roswell Park Cancer Institute).** 1994. Personal communication.
14. **Burhans, W. C., and J. A. Huberman.** 1994. DNA replication origins in animal cells: a question of context? Science **263:**639–640.
15. **Burhans, W. C., L. T. Vassilev, M. S. Caddle, N. H. Heintz, and M. L. DePamphilis.** 1990. Identification of an origin of bidirectional DNA replication in mammalian chromosomes. Cell **62:**955–965.
16. **Burhans, W. C., L. T. Vassilev, J. Wu, J. M. Sogo, F. N. Nallaseth, and M. L. DePamphilis.** 1991. Emetine allows identification of origins of mammalian DNA replication by imbalanced DNA synthesis, not through conservative nucleosome segregation. EMBO J. **10:**4351–4360.
17. **Caddle, M. S., and M. P. Calos.** 1992. Analysis of the autonomous replication behavior in human cells of the DHFR putative chromosomal origin of replication. Nucleic Acids Res. **20:**5971–5978.
18. **Caddle, M. S., R. H. Lussier, and N. H. Heintz.** 1990. Intramolecular DNA triplexes, bent DNA and DNA unwinding elements in the initiation region of an amplified dihydrofolate reductase replicon. J. Mol. Biol. **211:**19–33.
19. **Carothers, A. M., G. Urlaub, N. Ellis, and L. A. Chasin.** 1983. Structure of the dihydrofolate reductase gene in Chinese hamster ovary cells. Nucleic Acids Res. **11:**1997–2012.
20. **Carroll, S. M., M. L. DeRose, J. L. Kolman, G. H. Nonet, R. E. Kelly, and G. M. Wahl.** 1993. Localization of a bidirectional DNA replication origin in the native locus and in episomally amplified murine adenosine deaminase loci. Mol. Cell. Biol. **13:**2971–2981.
21. **Church, G. M., and W. Gilbert.** 1984. Genomic sequencing. Proc. Natl. Acad. Sci. USA **81:**1991–1995.
22. **Coverly, D., and R. A. Laskey.** 1994. Regulation of eukaryotic DNA replication. Annu. Rev. Biochem. **63:**745–776.
23. **Cox, L.** 1992. DNA replication in cell-free extract from *Xenopus* eggs is prevented by disrupting nuclear envelope function. J. Cell Sci. **101:**43–53.
24. **Decker, R. S., M. Yamaguchi, R. Possenti, and M. L. DePamphilis.** 1986. Initiation of simian virus 40 DNA replication in vitro: aphidicolin causes accumulation of early replicating intermediates and allows determination of the initial direction of DNA synthesis. Mol. Cell. Biol. **6:**3815–3825.
25. **DePamphilis, M. L.** 1993. Eukaryotic DNA replication: anatomy of an origin. Annu. Rev. Biochem. **62:**29–63.
26. **DePamphilis, M. L.** 1993. Origins of DNA replication in metazoan chromosomes. J. Biol. Chem. **268:**1–4.
27. **DePamphilis, M. L.** Specific labeling of newly replicated DNA. Methods Enzymol., in press.
28. **Diffley, J. F. X., J. H. Cocker, S. J. Dowell, and A. Rowley.** 1994. Two steps in the assembly of complexes at yeast replication origins in vivo. Cell **78:**303–316.
29. **Dijkwel, P. A., and J. L. Hamlin.** 1988. Matrix attachment regions are positioned near replication initiation sites, genes, and an interamplicon junction in the amplified dihydrofolate reductase domain of Chinese hamster vary cells. Mol. Cell. Biol. **8:**5398–5409.
30. **Dijkwel, P. A., and J. L. Hamlin.** 1992. Initiation of DNA replication in the *DHFR* locus is confined to the early S period in CHO cells synchronized with the plant amino acid mimosine. Mol. Cell. Biol. **12:**3715–3722.
31. **Dijkwel, P. A., J. P. Vaughn, and J. L. Hamlin.** 1994. Replication initiation sites are distributed widely in the amplified CHO dihydrofolate reductase domain. Nucleic Acids Res. **22:**4989–4996.
32. **Dinter-Gottlieb, G., and G. Kaufmann.** 1983. Aphidicolin arrest irreversibly impairs replicating SV40 chromosomes. J. Biol. Chem. **258:**3809–3812.
33. **Dobbs, D. L., W.-I. Shaiu, and R. M. Benbow.** 1994. Modular sequence elements associated with origin regions in eukaryotic chromosomal DNA. Nucleic Acids Res. **22:**2479–2489.
34. **Fang, F., and J. W. Newport.** 1993. Distinct roles of cdk2 and cdc2 in RP-A phosphorylation during the cell cycle. J. Cell Sci. **106:**983–994.
35. **Fox, M. H., D. J. Arndt-Jovin, T. M. Jovin, P. H. Baumann, and M. Robert-Nicoud.** 1991. Spatial and temporal distribution of DNA replication sites localized by immunofluorescence and confocal microscopy in mouse fibroblasts. J. Cell Sci. **99:**247–253.
36. **Gale, J. M., R. A. Tobey, and J. A. D'Anna.** 1992. Localization and DNA sequence of a replication origin in the rhodopsin gene locus of Chinese hamster cells. J. Mol. Biol. **224:**343–352.
37. **Gamper, H., N. Lehman, J. Piette, and J. E. Hearst.** 1985. Purification of circular DNA using benzoylated naphthoylated DEAE-cellulose. DNA **4:**157–164.
38. **Giacca, M. (International Centre for Genetic Engineering and Biotechnology, Trieste, Italy).** 1995. Personal communication.
39. **Giacca, M., L. Zentilin, P. Norio, S. Diviacco, D. Dimitrova, G. Contreas, G. Biamonti, G. Perini, F. Weighardt, S. Riva, and A. Falaschi.** 1994. Fine mapping of a replication origin of human DNA. Proc. Natl. Acad. Sci. USA **91:**7119–7124.
40. **Gilbert, D. M., and S. N. Cohen.** 1987. Bovine papilloma virus plasmids replicate randomly in mouse fibroblasts throughout S-phase of the cell cycle. Cell **50:**59–68.
41. **Gilbert, D. M., R. Losson, and P. C. Chambon.** 1992. Ligand dependence of estrogen receptor induced changes in chromatin structure. Nucleic Acids Res. **20:**4525–4531.
42. **Gilbert, D. M., H. Miyazawa, F. S. Nallaseth, J. M. Ortega, J. J. Blow, and M. L. DePamphilis.** 1994. Site-specific DNA replication in metazoan chromosomes and the role of nuclear organization. Cold Spring Harbor Symp. Quant. Biol. **58:**475–485.
43. **Gilbert, D. M., A. Neilson, H. Miyazawa, M. L. DePamphilis, and W. C. Burhans.** Mimosine arrests DNA synthesis at replication forks by inhibiting deoxyribonucleotide metabolism. J. Biol. Chem., in press.
44. **Gray, J. W., and P. Coffino.** 1979. Cell cycle analysis by flow cytometry. Methods Enzymol. **58:**233–248.
45. **Haase, S. B., S. S. Heinzel, and M. P. Calos.** 1994. Transcription inhibits the replication of autonomously replicating plasmids in human cells. Mol. Cell. Biol. **14:**2516–2526.
46. **Handeli, S., A. Klar, M. Meuth, and H. Cedar.** 1989. Mapping replication units in animal cells. Cell **57:**909–920.
47. **Harland, R., and L. Misher.** 1988. Stability of RNA in developing *Xenopus* embryos and identification of a destabilizing sequence in TFIIIA messenger

RNA. Development **102:**837–852.
48. **Heintz, N. H., and B. Stillman.** 1988. Nuclear synthesis in vitro is mediated via stable replication forks assembled in a temporarily specific fashion in vivo. Mol. Cell. Biol. **8:**1923–1931.
49. **Hozák, P., A. B. Hassan, D. A. Jackson, and P. R. Cook.** 1993. Visualization of replication factories attached to nucleoskeleton. Cell **73:**361–373.
50. **Hyrien, O., and M. Méchali.** 1993. Chromosomal replication initiates and terminates at random sequences but at regular intervals in the ribosomal DNA of *Xenopus* early embryos. EMBO J. **12:**4511–4520.
51. **Ishimi, Y., K. Matsumoto, and R. Ohba.** 1994. DNA replication from initiation zones of mammalian cells in a model system. Mol. Cell. Biol. **14:**6489–6496.
52. **Jackson, D. A., and P. R. Cook.** 1986. Replication occurs at a nucleoskeleton. EMBO J. **5:**1403–1413.
53. **Jenkins, H., T. Holman, C. Lyon, B. Lane, R. Stick, and C. Hutchison.** 1993. Nuclei that lack a lamina accumulate karyophilic proteins and assemble a nuclear matrix. J. Cell Sci. **106:**275–285.
54. **Kelly, R. E., M. L. DeRose, B. W. Draper, and G. M. Wahl.** 1994. Identification of an origin of bidirectional DNA replication in the coding region of the ubiquitously expressed mammalian CAD gene. Submitted for publication.
55. **Kill, I. R., J. M. Bridges, K. H. S. Campbell, G. Maldonado-Codina, and C. J. Hutchison.** 1991. The timing of the formation and usage of replicase clusters in S-nuclei of human diploid fibroblasts. J. Cell Sci. **100:**869–876.
56. **Kimelman, D., M. Kirschner, and T. Scherson.** 1987. The events of the midblstula transition in *Xenopus* are regulated by changes in the cell cycle. Cell **48:**399–407.
57. **Kitsberg, D., S. Selig, I. Keshet, and H. Cedar.** 1993. Replication structure of the human β-globin gene domain. Nature (London) **366:**588–590.
58. **Kornberg, A., and T. Baker.** 1992. DNA replication. W. H. Freeman and Co., New York.
59. **Krysan, P. J., J. G. Smith, and M. P. Calos.** 1993. Autonomous replication in human cells of multimers of specific human and bacterial DNA sequences. Mol. Cell. Biol. **13:**2688–2696.
60. **Kubota, Y., and H. Takisawa.** 1993. Determination of initiation of DNA replication before and after nuclear formation in *Xenopus* egg cell free extract. J. Cell Biol. **123:**1321–1331.
61. **Leibovichi, M., G. Monod, J. Géraudie, R. Bravo, and M. Méchali.** 1992. Nuclear distribution of PCNA during embryonic development in *Xenopus laevis*: a reinvestigation of early cell cycles. J. Cell Sci. **102:**63–69.
62. **Leno, G. H., C. S. Downes, and R. A. Laskey.** 1992. The nuclear membrane prevents replication of human G2 nuclei but not G1 nuclei in *Xenopus* egg extract. Cell **69:**151–158.
63. **Leu, T.-H., and J. L. Hamlin.** 1989. High-resolution mapping of replication fork movement through the amplified dihydrofolate reductase domain in CHO cells by in-gel renaturation analysis. Mol. Cell. Biol. **9:**523–531.
64. **Liang, C., and S. A. Gerbi.** 1994. Analysis of an origin of DNA amplification in *Sciara coprophila* by a novel three dimensional gel method. Mol. Cell. Biol. **14:**1520–1530.
65. **Lin, S., and D. Kowalski.** 1994. DNA helical instability facilitates initiation at the SV40 replication origin. J. Mol. Biol. **235:**496–507.
66. **Little, R. D., T. H. K. Platt, and C. L. Schildkraut.** 1993. Initiation and termination of DNA replication in human rRNA genes. Mol. Cell. Biol. **13:**6600–6610.
67. **Mahbubani, H. M., T. Paull, J. K. Elder, and J. J. Blow.** 1992. DNA replication initiates at multiple sites on plasmid DNA in *Xenopus* egg extract. Nucleic Acids Res. **20:**1457–1462.
68. **Masukata, H., H. Satoh, C. Obuse, and T. Okazaki.** 1993. Autonomous replication of human chromosomal DNA fragments in human cells. Mol. Biol. Cell **4:**1121–1132.
69. **McKnight, S. L., and O. L. Miller.** 1977. Electron microscopic analysis of chromatin replication in the cellular blastoderm *Drosophila melanogaster* embryo. Cell **12:**795–804.
70. **McNeil, P. L., R. F. Murphy, F. Lanni, and D. L. Taylor.** 1984. A method for incorporating macromolecules into adherent cells. J. Cell Biol. **98:**1556–1564.
71. **Melera, P. W., J. P. Davide, and H. Oen.** 1988. Antifolate-resistant Chinese hamster cells. J. Biol. Chem. **263:**1978–1990.
72. **Montag, M., H. Spring, and M. F. Trendelenburg.** 1988. Structural analysis of the mitotic cycle in pre-gastrula *Xenopus* embryos. Chromosoma **96:**187–196.
73. **Nakamura, H., T. Morita, S. Masaki, and S. Yoshida.** 1984. Intracellular localization and metabolism of DNA polymerase alpha in human cells visualized with monoclonal antibody. Exp. Cell Res. **151:**123–133.
74. **Nakayasu, H., and R. Berezney.** 1989. Mapping replicational sites in the eucaryotic cell nucleus. J. Cell Biol. **108:**1–11.
75. **Natale, D. A., R. M. Umek, and D. Kowalski.** 1993. Ease of DNA unwinding is a conserved property of yeast replication origins. Nucleic Acids Res. **21:**555–560.
76. **Nethanel, T., and G. Kaufmann.** 1990. Two DNA polymerases may be required for synthesis of the lagging DNA strand of simian virus 40. J. Virol. **64:**5912–5918.
77. **Newport, J.** 1987. Nuclear reconstitution in vitro: stages of assembly around protein-free DNA. Cell **48:**205–217.
78. **Newport, J. W., K. L. Wilson, and W. G. Dunphy.** 1990. A lamin-independent pathway for nuclear envelope assembly. J. Cell Biol. **111:**2247–2259.
79. **O'Keefe, R. T., S. C. Henderson, and D. L. Spector.** 1992. Dynamic organization of DNA replication in mammalian cell nuclei: spatially and temporally-defined replication of chromosome specific α-satellite sequences. J. Cell Biol. **116:**1095–1100.
80. **Orr-Weaver, T. L.** 1991. Drosophila chorion genes: cracking the eggshell's secrets. Bioessays **13:**97–105.
81. **Prioleau, M.-N., J. Huet, A. Sentenac, and M. Méchali.** 1994. Competition between chromatin and transcription complex assembly regulates gene expression during early development. Cell **77:**439–449.
82. **Shinomiya, T., and S. Ina.** 1991. Analysis of chromosomal replicons in early embryos of *Drosophila* melanogaster by two-dimensional gel electrophoresis. Nucleic Acids Res. **19:**3935–3941.
83. **Shinomiya, T., and S. Ina.** 1994. Mapping an initiation region of DNA replication at a single-copy chromosomal locus in *Drosophila melanogaster* cells by two-dimensional gel methods and PCR-mediated nascent-strand analysis: multiple replication origins in a broad zone. Mol. Cell. Biol. **14:**7394–7403.
84. **Sleeman, A. M., G. H. Leno, A. D. Mills, M. P. Fairman, and R. A. Laskey.** 1992. Patterns of DNA replication in Drosophila polytene nuclei replicating in *Xenopus* egg and oocyte extract. J. Cell Sci. **101:**509–515.
85. **Snapka, R. M., C. G. Shin, P. A. Permana, and J. Strayer.** 1991. Aphidicolin-induced topological and recombinational events in SV40. Nucleic Acids Res. **19:**5065–5072.
86. **Stick, R., and P. Hausen.** 1985. Changes in the nuclear lamina composition during early development of *Xenopus laevis*. Cell **41:**191–200.
87. **Taira, T., S. M. M. Iguchi-Ariga, and H. Ariga.** 1994. A novel DNA replication origin identified in the human heat shock protein 70 gene promoter. Mol. Cell. Biol. **14:**6386–6397.
88. **Tasheva, E. S., and D. J. Roufa.** 1994. A mammalian origin of bidirectional DNA replication within the Chinese hamster RPS14 locus. Mol. Cell. Biol. **14:**5628–5635.
89. **Tasheva, E. S., and D. J. Roufa.** 1994. Densely methylated DNA islands in mammalian chromosomal replication origins. Mol. Cell. Biol. **14:**5636–5644.
90. **Trask, B. J., and J. L. Hamlin.** 1989. Early dihydrofolate reductase gene amplification events in CHO cells usually occur on the same chromosome arm as the original locus. Genes Dev. **3:**1913–1925.
91. **Virta-Pearlman, V. J., P. H. Gunaratne, and A. C. Chinault.** 1993. Analysis of a replication initiation sequence from the adenosine deaminase region of the mouse genome. Mol. Cell. Biol. **13:**5931–5942.
92. **Wang, T. S.-F.** 1991. Eukaryotic DNA polymerases. Annu. Rev. Biochem. **60:**513–552.
93. **Weaver, D. T., S. C. Fields-Berry, and M. L. DePamphilis.** 1985. The termination region for SV40 DNA replication directs the mode of separation for the two sibling molecules. Cell **41:**565–575.
94. **Wolffe, A. P.** 1994. The role of transcription factors, chromatin structure and DNA replication in 5S RNA gene regulation. J. Cell. Sci. **107:**2055–2063.
95. **Wu, C., M. Zannis-Hadjopoulos, and G. B. Price.** 1993. In vivo activity for initiation of DNA replication resides in a transcribed region of the human genome. Biochim. Biophys. Acta **1174:**258–268.
96. **Yamamoto, S., T. Takahashi, and A. Matsukage.** 1984. Tight association of DNA polymerase alpha with granular structures in the nuclear matrix of chick embryo cells: immunocytochemical detection with monoclonal antibody against DNA polymerase alpha. Cell Struct. Funct. **9:**83–90.
97. **Yoon, Y., J. A. Sanchez, C. Brun, and J. A. Huberman.** 1995. Mapping of replication initiation sites in human ribosomal DNA by nascent-strand abundance analysis. Mol. Cell. Biol. **15:**2482–2489.